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Leader sequence of a plant ribosomal protein gene with complementarity to the 18S rRNA triggers in vitro cap-independent translation

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Abstract **Cap-independent translation (CIT) occurs at the leader sequences of uncapped plant viral RNAs, but also at a number of normally capped cellular mRNAs and has been correlated with sequence complementarity to 18S rRNA. The ribosomal protein S18 (RPS18) is a component of the small ribosomal subunit and is encoded by three gene copies (A, B, and C) in the *Arabidopsis thaliana* genome. The *RPS18C* mRNA was most abundant and contained a short 5′ untranslated region of 84 bp that is complementary to a novel putative interaction site at the 3′ end of the 18S rRNA. The *RPS18C* leader mediated CIT as demonstrated by dicistronic constructs consisting of luciferase and chloramphenicol acetyl transferase reporter genes in an in vitro wheat germ extract system. CIT was rapidly inhibited upon addition of an oligonucleotide that competed for the 18S rRNA site complementary to the *RPS18C* leader and interfered with polysome assembly at the transcript.**

Keywords: Cap-independent translation; 18S rRNA; Internal ribosome entry site (IRES); Ribosomal protein; *Arabidopsis thaliana*

1. Introduction

The ribosome scanning model describing eukaryotic translation initiation is based on the interaction of multiple initiation factors with common *cis*-acting elements in messenger RNAs (5′ terminal 7-methylguanosine [cap], polyadenylation tail [poly(A)], and initiation codon region). First, protein complexes direct the loading of the small ribosomal subunit (40S) onto eukaryotic messengers after the mRNA 5′ cap and cap-associated factors have been recognized. The 40S ribosomal subunit then joins the messenger and scans the 5′ untranslated region (UTR) in search for an AUG codon in a favorable sequence context. At this point, the large ribosomal subunit is added to the complex and protein synthesis starts [1]. In prokaryotes, the recruitment of the small ribosomal subunit (30S) is basically an RNA–RNA interaction. The 30S subunit recognizes the mRNA by direct base pairing between the 3′ end of the 16S rRNA and the complementary sequences on the mRNA (Shine-Dalgarno elements), immediately upstream of the AUG start codon [2].

Sequence elements in the 5′ non-coding regions of some eukaryotic messengers can initiate cap-independent translation (CIT) by internal ribosome entry through complementarity with the 18S rRNA at several positions [3,4]. Internal ribosome entry sites (IRESs) were found first in uncapped picornaviral mRNAs [5] and, later, in a number of capped cellular messengers in yeast, *Drosophila*, and mammals [6]. This alternative mechanism has been postulated to control selective translation of mRNAs during growth, differentiation, and in stress responses. According to the ribosome filter hypothesis, the rate of polypeptide production might be regulated by differential binding of particular mRNAs to the 18S rRNA or to ribosomal proteins of the eukaryotic 40S ribosomal subunits [7]. The plant translational apparatus can mediate CIT on viral IRESs [8,9]. Recently, the 5′UTR of the maize heat shock protein gene *HSP101* was shown to exhibit IRES activity [10].

We demonstrate CIT at the leader of the *Arabidopsis RPS18C* gene and provide experimental proof that the process relies on leader sequence complementarity to a novel site at the 3′ end of the 18S rRNA.

2. Materials and methods

2.1. Reverse transcriptase polymerase chain reaction (RT-PCR)

mRNA was isolated from plant material, grown in vitro on GM medium [11], with the QuickPrep mRNA Purification Kit (GE-Heathcare, Little Chalfont, UK). cDNA was synthesized with the Superscript Preamplification System for First Strand cDNA Synthesis (Invitrogen, Carlsbad, CA). Multiplex PCR oligonucleotides were described [12] and PCR products were separated on polyacrylamide sequencing gels that were dried and exposed overnight. The distinct amplicons were quantified with a PhosphorImager using the ImageQuant 4.1 software (GE-Heathcare).

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Abbreviations: CAT, chloramphenicol acetyl transferase; CIT, cap-independent translation; hp, hairpin; IRES, internal ribosome entry site; LUC, luciferase; ORF, open reading frame; poly(A), polyadenylation; RT-PCR, reverse transcriptase-polymerase chain reaction; 5′UTR, 5′untranslated region

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2.2. Plasmid construction

The monocistronic leader-chloramphenicol acetyl transferase (CAT) sequence was constructed in a T7/SP6 in vitro transcription vector as the translational fusion of the *RPS18C* leader to the *CAT* open reading frame (ORF) in pFM169 [13]. In that construct, the ORF was preceded by the tobacco mosaic virus leader, Ω, and followed by a synthetic poly(A) sequence. In a first step, the leader sequence was amplified by PCR from a *RPS18C* genomic clone with primers 5′-CCTCTTTTGGGATCCTCACTCTC-3′ and 5′-CTAATTACCATGGTGATTAGCAGAG-3′, hereby creating a *Bam*HI and *Nco*I restriction site (underlined) at the 5′ end of the leader and at the AUG start codon, respectively. In the second step, the 5′ end of the *CAT* ORF was amplified from pFM169 with primers 5′-ACTATTCTAGCCATGGAGAA-3′ and 5′-CCATACGGAATTCCGGATGA-3′, introducing a *Nco*I site at the translation start codon of *CAT* and covering the existing *Eco*RI site at position 286 in pFM169, respectively. Both fragments were purified, restriction digested, and ligated. The resulting *Bam*HI/*Eco*RI fragment was cloned in pFM169. The desired clone was sequence verified. The monocistronic luciferase (*LUC*) transcript was synthesized from the *pT3/T7-LUC* expression vector (Clontech Laboratories, Palo Alto, CA).

The *LUC-leader-CAT* construct was made by inserting a 1.9-kb *Bam*HI fragment from *pT3/T7-LUC*, covering the entire *LUC* gene, in front of the *leader-CAT* cassette digested with *Bam*HI, resulting in the *LUC-IRES-CAT* construct. The negative control *LUC-antisense leader-CAT* was constructed in several steps. The blunt-ended *Bam*HI–*LUC* fragment (see above) was first cloned into the blunt-ended *Sac*I site of pFM136 [14], a pGEM-3Z vector derivative containing the *CAT* ORF. The poly(A) sequence from pFM169 was inserted as an *Xba*I–*Hind*III fragment at the 3′ end of the *CAT* ORF yielding the intermediate vector *LUC-no_spacer-CAT*. In a second step, blunt-ended *Sma*I/*Nco*I fragments isolated from the *LUC-leader-CAT* construct containing the entire *RPS18C* leader were cloned into the *Sma*I-digested *LUC-no_spacer-CAT*. A sequence-validated clone was selected that contained the antisense *RPS18C* leader sequence between both ORFs and resulted in *LUC-SERI-CAT*.

The hairpin (hp) construct, *hpLUC-leader-CAT*, was made by annealing two complementary oligonucleotides, 5′-GATCCAGCTTGGGCCGTGGTGGAGCTTCCACCACGGCCCTTCGAG-3′ and 5′-GATCCTCGAAGGGCCGTGGTGGAAGCTCCACCACGGCCCAAGCTG-3′, producing *Bam*HI sites at both termini. The double-stranded loop was ligated in partially *Bam*HI-digested *LUC-leader-CAT*, yielding *hpLUC-IRES-CAT*.

2.3. In vitro transcription and translation

All transcripts were synthesized in vitro from 1 μg *Hin*dIII-linearized plasmid DNA template with T7 RNA polymerase (Ampliscribe High Yield Transcription Kit; Epicentre Technologies, Madison, WI), except for the monocistronic *LUC* transcript that was synthesized with T3 RNA polymerase from *Sma*I-linearized *pT3/T7-LUC*. After DNaseI treatment, RNA was purified by precipitation with 1 volume 5 M ammonium acetate, centrifuged, washed with 70% ethanol, and dissolved in RNase-free water. RNA was checked for quality by agarose gel electrophoresis and quantified by spectrophotometry.

In vitro protein synthesis was performed in wheat germ extract (Promega, Madison, WI). Reactions included 3 pmoles in vitro synthesized RNA, RNasin ribonuclease inhibitor (Promega), with a final concentration of 73 mM potassium acetate and 2.1 mM magnesium acetate. Translation products were labeled by incorporation of L-[^{35}S]-methionine (GE-Heathcare) and analyzed after a 45-min separation by SDS–PAGE. The 12% polyacrylamide gels were fixed in 10% acetic acid, dried, and analyzed with a PhosphorImager and ImageQuant 4.1 software (GE-Heathcare). Enzymatic activities of LUC and CAT were quantified with the respective enzyme assay system (both from Promega) and a liquid scintillation counter (1209 Rackbeta, LKB, and TopCount .NXT, Packard, respectively).

2.4. Oligoribonucleotide competition assays

Oligoribonucleotides were purchased from Genset (Paris, France). All competition assays were repeated 2–4 times. Slight discrepancies between the inhibitory concentration of oligoribonucleotides are due to batch-to-batch wheat germ extract heterogeneity.

2.5. RNA gel blot analysis

Following in vitro translation reactions, mRNA integrity was verified by RNA gel blot analysis as described [12]. Samples were extracted twice with phenol/chloroform and precipitated with 1 volume of 5 M ammonium acetate before loading onto formamide gels.

2.6. Sucrose density gradient centrifugation

To investigate monosome-polysome profiles, 15 μl of each in vitro translation reaction was loaded onto a linear 10–45% sucrose density gradient (25 mM Tris–HCl (pH 7.6), 100 mM KCl, and 5 mM $MgCl_2$). After centrifugation in a SW41 rotor (Beckman Coulter, Fullerton, CA) at 38000 rpm for 1 h at 4 °C, fractions were collected from the bottom of the tubes and absorbance measured at 260 nm.

2.7. Accession numbers

The GenBank/EMBL accession number for the genomic sequence of *RPS18C* is Z28962. Five cDNA clones (T22876, H76969, T44679, H76716, and H76558) were isolated corresponding to this gene, all of them including the mRNA_15 motif. The sequence of the *RPS18C* leader shown in Fig. 2A corresponds to the longest expressed sequence tag clone identified when sequence analysis was performed (T22876).

3. Results

3.1. The RPS18C gene copy is most abundantly transcribed

In *Arabidopsis thaliana*, the S18 ribosomal protein is encoded by three genes, *RPS18A* (At1g22780.1), *RPS18B* (At1g34030.1), and *RPS18C* (At4g09800.1), that are distributed across the genome [12]. We determined the transcriptional contribution of the three gene copies in various tissues via multiplex RT-PCR. To distinguish between the three transcripts, PCRs were performed with a common [^{32}P]-end-labeled oligonucleotide corresponding to an identical sequence in their third exon in combination with gene-specific oligonucleotides anchored in their respective 5′UTRs (Fig. 1A). The PCRs resulted in the amplification of three products with distinct size each specific to a single *RPS18* gene. Melting temperature of the common primer was the lowest ($T_m = 41$ °C; $\Delta G = -35.4$ kcal/mol) and determined the kinetics of the three PCRs within the same sample. The PCR product concentrations were analyzed at a fixed time point during the linear phase of the reaction (18 cycles) and were representative for their initial concentration. In all wild-type tissues tested, such as roots (Fig. 1B, lane 1), seedlings at different ages (Fig. 1B, lanes 2-5), young leaves (Fig. 1B, lane 6), expanded leaves (Fig. 1B, lane 7), and heart-stage embryos (data not shown), the relative contribution of the three transcripts to the pool of S18 protein encoding mRNA was remarkably stable: on average 27%, 16% and 57% for *RPS18A*, *RPS18B*, and *RPS18C*, respectively (Fig. 1B).

The phenotype of an insertion mutant in the *RPS18A* gene copy had previously been studied and resulted in a narrow and pointed first leaf shape (*pfl*) and reduced biomass [12]. Multiplex RT-PCR on expanded leaves of the *pfl* mutant showed a 10-fold reduction of the *RPS18A* transcript (Fig. 1B, lanes 7 and 8) and no increase in expression of the *RPS18B* and *RPS18C* gene copies, indicating absence of transcriptional compensation.

3.2. The RPS18C leader contains sequence complementary to the 3′ end of the 18S rRNA

By looking for *cis*-acting elements potentially responsible for the differential expression of the *RPS18* genes, we identified a

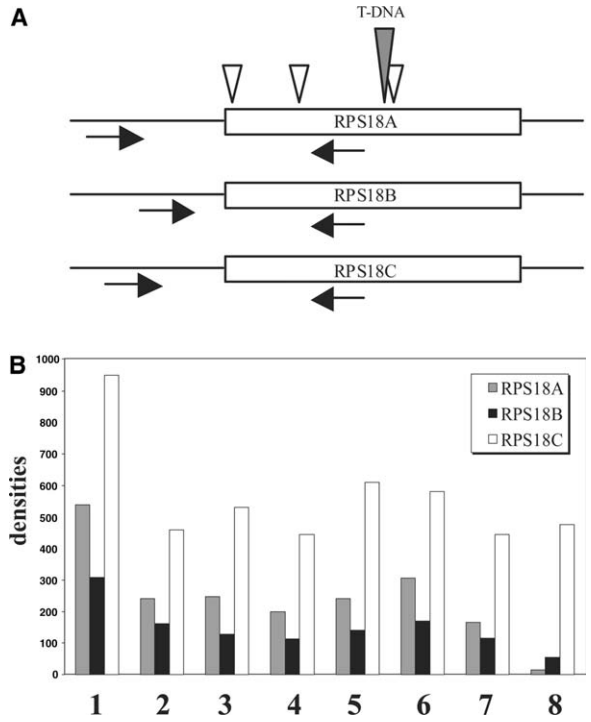

Fig. 1. Expression analysis of the *RPS18* genes. (A) Schematic representation of the position of the RT-PCR primers (black arrows). Open and closed triangles represent intron positions in all three genes and the T-DNA insertion site in *RPS18A*. (B) Relative quantification of *RPS18* transcripts. Graph shows the quantified signal for each PCR product in wild-type tissues (lane 1, roots; lane 2, 5-day-old seedlings; lane 3, 12-day-old seedlings; lane 4, 19-day-old seedlings; lane 5, flowering plants; lane 6, young leaves; lane 7, mature leaves; and lane 8, mature leaves of *pfl* homozygous mutant.

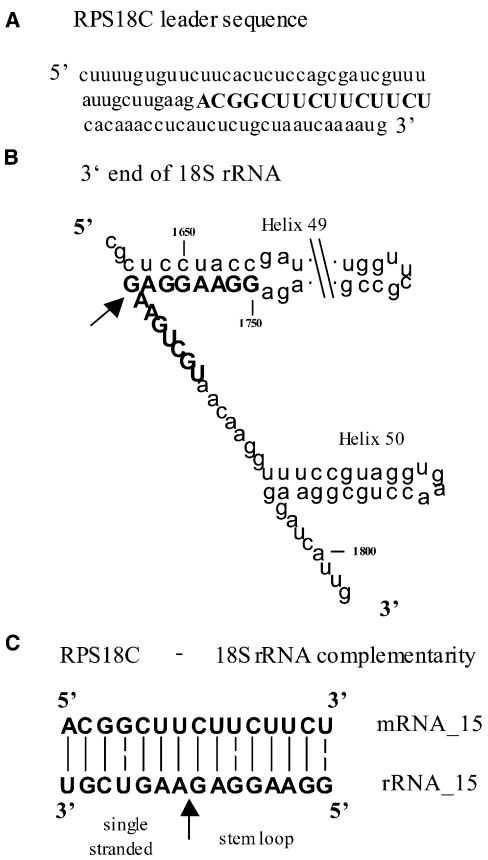

Fig. 2. Sequence complementarity between the *RPS18C* leader and the 3′ end of the 18S rRNA. (A) Primary sequence of the *RPS18C* leader. Translation start codon is included and sequence complementary to 18S rRNA (mRNA_15) is highlighted (bold and uppercase). (B) Predicted primary and secondary structure of the 3′ end of the 18S rRNA. The potential interaction site (rRNA_15) with the *RPS18C* leader is highlighted (bold and uppercase). (C) Base pairing between mRNA_15 and rRNA_15. Full and interrupted lines mark Watson–Crick and G-U wobble pairs, respectively. The junction between the portion of rRNA_15 part of the stem-loop structure and the unpaired bases in the 18S rRNA is indicated by an arrow.

stretch of 15 nucleotides in the 5′UTR of *RPS18C* (Fig. 2A) that is complementary to the 3′ end of the *Arabidopsis* 18S rRNA (Fig. 2B and C). The stretch of 15 nucleotides at position 44–58 in the *RPS18C* leader (mRNA_15 in Fig. 2C) is fully complementary to a region near the 3′ end of the *Arabidopsis* 18S rRNA sequence at position 1750–1764 (rRNA_15 in Fig. 2C). The last eight bases of mRNA_15 match a GA-rich sequence within the stem of helix 49, and the first seven bases match single-stranded sequences between helices 49 and 50 of the 18S rRNA two-dimensional model described by Van de Peer et al. [15] (Fig. 2B). Although this region in the 18S rRNA is highly conserved in eukaryotes, it has never been proposed as a putative interaction site with mRNAs.

3.3. The RPS18C leader mediates CIT in vitro

Complementarity of sequences to the leaders of eukaryotic mRNAs to certain regions of the 18S rRNA has been shown to allow CIT of these mRNAs [16]. By insertion of the *RPS18C* leader between two ORFs of a dicistronic construct it is possible to test for CIT [17]. We constructed a dicistronic vector with the *LUC*-coding sequence as the first ORF and the *CAT*-coding sequence as the second ORF preceded by the *RPS18C* leader of 84 bp. If the leader region mediates CIT, the *CAT* ORF should be translated from this *LUC-leader-CAT* mRNA independently from the *LUC* ORF. We cloned a stable hp in the leader of the first reporter gene (*hpLUC-IRES-CAT*) to reduce translation from the first ORF in a cell-free system. Consequently, if translation of the second ORF remained unaffected, it would unambiguously show internal ribosome entry activity at the IRES (*RPS18C* leader) sequence in comparison to a construct without stemloop (*LUC-IRES-CAT*). As a negative control, the antisense sequence of the *RPS18C* leader, referred to as SERI, was fused to the start codon of the *CAT* reporter gene (*LUC-SERI-CAT*). These constructs were transcribed and translated in an in vitro wheat germ extract, labeled with ^{35}S-methionine and visualized on polyacrylamide gels (Fig. 3A). CAT enzyme was produced from the dicistronic constructs *LUC-IRES-CAT* and *hpLUC-IRES-CAT*, but not from the negative control. With the *hpLUC-IRES-CAT* construct, LUC enzyme activity was reduced at least 10-fold without affecting the level of CAT enzyme activity, indicating that the CAT enzyme produced in this construct was not due to a readthrough mechanism from the first ORF but originated from internal ribosome entry at the IRES (*RPS18C* leader) sequence (Fig. 3A and C). RNA gel blot analysis was done after in vitro translation and showed transcripts to be intact, indicating that CAT translation was not caused by truncated transcripts as a result

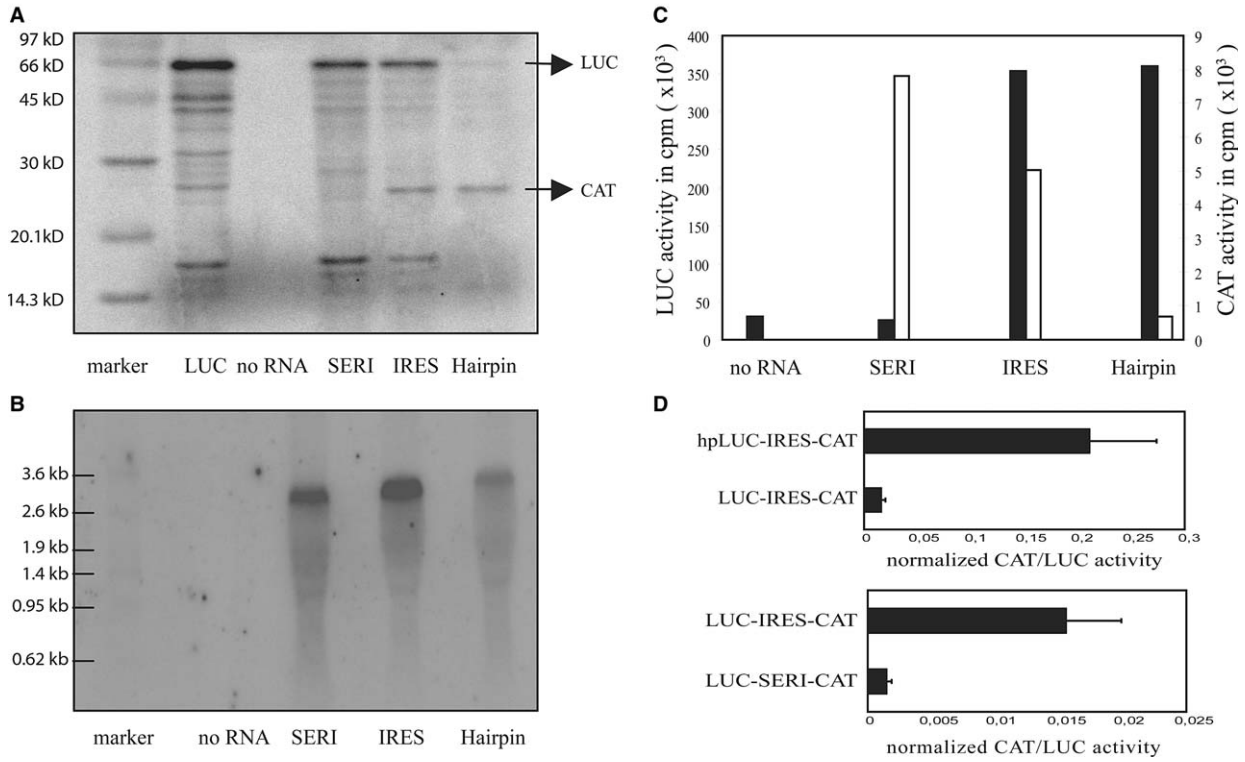

Fig. 3. In vitro IRES activity of the *RPS18C* leader. (A) Analysis of the translational products. Extracts were programmed with the indicated monocistronic transcripts derived from *pT3/T7-LUC* (LUC), and the dicistronic constructs *LUC-SERI-CAT* (SERI), *LUC-IRES-CAT* (IRES) and *hpLUC-IRES-CAT* (Hairpin), and synthesized polypeptides labeled with ^{35}S-methionine were separated by SDS–PAGE. (B) RNA gel blot analysis of wheat germ extract after translation of the dicistronic transcripts (*CAT* DNA probe). Samples are identical to those yielding the results pictured in Fig. 3A. (C) Enzymatic activities of LUC (open bar) and CAT (black bar) corresponding to the samples pictured in Fig. 3A. (D) Normalized CAT versus LUC enzymatic activity (three repeats).

of fragmentation or degradation (Fig. 3B). LUC and CAT activities were measured in similar repetitive assays and showed statistically relevant differences between *hpLUC-IRES-CAT* and *LUC-IRES-CAT* (Fig. 3D, upper panel) and between *LUC-IRES-CAT* and *LUC-SERI-CAT* (Fig. 3D, lower panel), as seen by normalized CAT/LUC activity. From the graphs, it is clear that CAT and LUC activities are independent (Fig. 3C and D). In conclusion, these data indicate IRES activity of the *RPS18C* leader sequence.

3.4. Complementarity between the leader and 18S rRNA is important for CIT

The exceptionally long stretch (15 nucleotides) of complementarity between the *RPS18C* leader and the 18S rRNA suggests that the two molecules might interact via base pairing. We investigated this putative interaction via in vitro translation competition experiments in which increasing amounts of oligoribonucleotides were added to wheat germ extract together with an uncapped transcript that coded for the *CAT* reporter gene preceded by the *RPS18C* leader. The oligoribonucleotides were designed to be complementary to different portions of the rRNA_15 motif in the 18S rRNA: OligoRiboNucleotide 1 (acronym ORN1; 5′-ACGACUUCUCCUUCCUCU-3′, $\Delta G = -34.3$ kcal/mol) that potentially hybridizes with segment 1747–1764 that spans the entire rRNA_15 motif; ORN2 (5′-UUGUUACGACUU-3′, $\Delta G = -16.4$ kcal/mol) with segment 1758–1769 that spans the 3′ portion of the motif positioned between helix 49 and helix 50 and part of a single-stranded stretch; and ORN3 (5′-UCUCCUUCCUCU-3′, $\Delta G = -21.6$ kcal/mol) that overlaps with the 5′ portion of the motif part in the stem of helix 49 (Fig. 4A). In addition, an oligoribonucleotide consisting of an arbitrarily chosen GAUC triplet (5′-GAUCGAUCGAUC-3′) was also included as a negative control. ORN1 and ORN2 dramatically inhibited in vitro translation, while equimolar amounts of the control oligoribonucleotide did not (Fig. 4B). Interestingly, ORN3 targeting the stem of helix 49 had also no effect (Fig. 4B). These results suggest that CIT inhibition correlates with the size of the overlap between the oligoribonucleotides and the single-stranded stretch in rRNA_15. This observation could be explained by the fact that the portion of the motif locked in the helix stem is not accessible for molecular hybridization with the competing oligoribonucleotides. Nevertheless, the same portion could be involved in strand displacement mechanisms initiated at the 3′ end of the motif. Importantly, in similar reactions, ORN1 had no effect on the translation of naturally capped brome mosaic virus transcripts, indicating that, when it occurred, translation inhibition was specific to uncapped transcripts (data not shown).

To rule out that decreased translation resulted from RNA degradation, the integrity of the transcript was verified by Northern blot analysis at the end of the in vitro incubation in wheat germ extract. Only full-length *RPS18C leader-CAT* mRNA were detected, and higher amounts were present in samples where translation was inhibited (Fig. 4C), suggesting

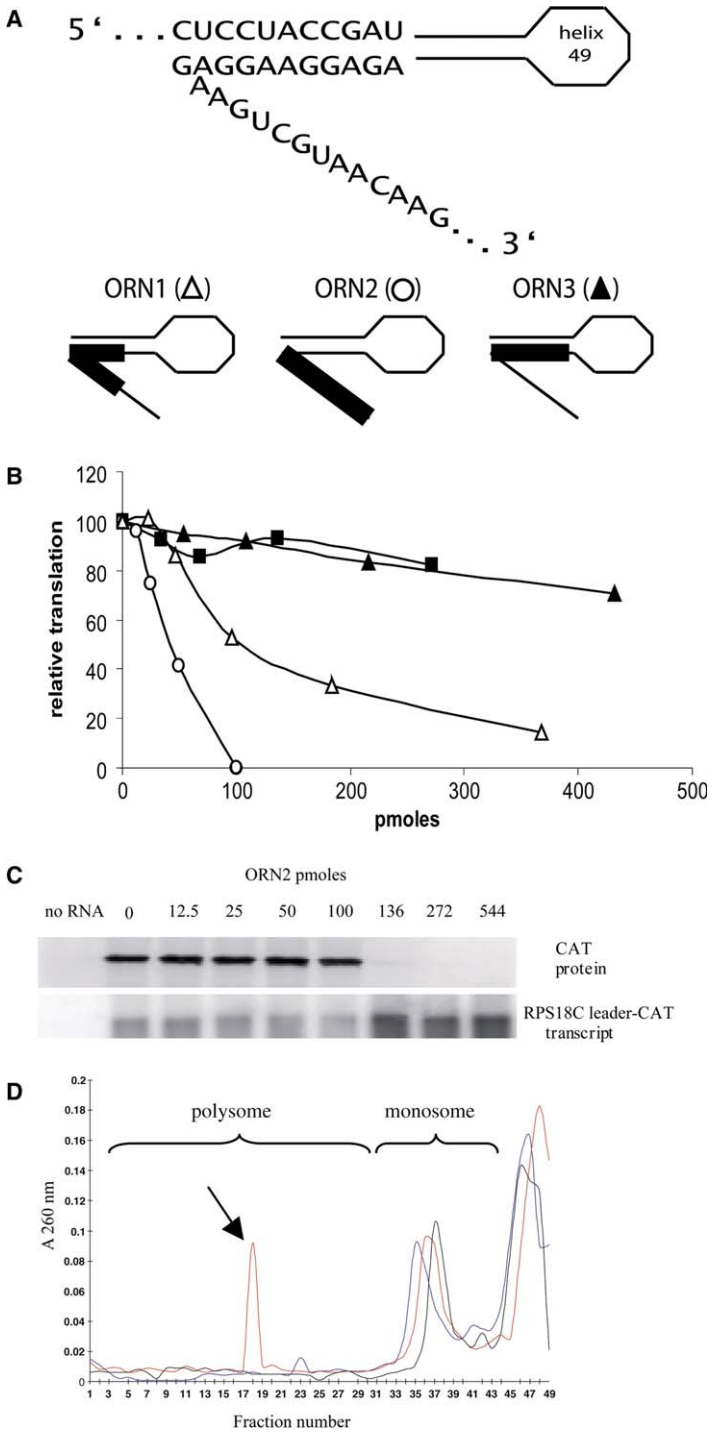

Fig. 4. Oligoribonucleotide competition experiments in a wheat germ translation system. (A) rRNA_15 motif embedded partially in helix 49 of the 40S ribosomal subunit. Thick segments mark the sequence complementary to ORN1, ORN2 or ORN3 in the 18S rRNA (see Fig. 2B). (B) CAT protein synthesis. The graph shows the competition effect of increasing amounts of each ORN measured by the translation efficiency of uncapped *RPS18C leader-CAT* transcripts. Relative radiolabeled CAT protein levels are expressed in percent in comparison to the reference control with no added oligoribonucleotide. Black square, random oligonucleotide; open triangle, ORN1; open circle, ORN2; and black triangle, ORN3. (C) Competition assay with increasing amounts of ORN2, showing the translation products (upper part) and the intact transcripts after RNA gel blot analysis on the same samples (lower part). (D) Polysome profiles of the reactions performed in (B), after fractionation in a linear 10–45% sucrose density gradient. Black line, negative control (no RNA, with 272 pmoles ORN2); red line, *RPS18C leader-CAT* transcript with no ORN; blue line, *RPS18C leader-CAT* with 272 pmoles ORN2. Arrow indicates polysome assembly.

that actively translated transcripts forming polyribosomes had been preferentially removed from the aqueous solution during RNA purification. To confirm this observation, ribosomal fractions were separated in sucrose gradients after in vitro translation of the *RPS18C leader-CAT* mRNA with or without inhibitory level of ORN2 (Fig. 4D). The ribosomal profiles

clearly showed that the 18S rRNA-competing oligonucleotide prevents translation by blocking polysome assembly.

4. Discussion

Complementary sequences to 18S rRNA occur frequently in eukaryotic mRNAs in sense or antisense orientation and are positioned in their leaders or coding sequences. rRNA-like sequences might have spread throughout the eukaryotic genome and their presence in primary transcripts might differentially affect translation through interaction with rRNA or ribosomal proteins [3]. Four short sequences with high complementarity to 18S rRNA occur in hundreds of eukaryotic mRNAs [3]; however, their position in the 18S rRNA differ from the sequence identified in this work. These short sequences have been shown to bind to 18S rRNA as illustrated for a 9-nucleotide sequence in the leader of the Gtx homeodomain transcription factor for which internal ribosome entry was demonstrated. Hence, complementary sequences to 18S rRNA might serve as *cis*-regulatory elements in translation [18]. Sequence complementarity to the middle part of the 18S rRNA has been identified in 5′UTR of viral transcripts. These sequence elements were capable of enhancing CIT of reporter gene constructs in eukaryotic in vitro translation systems of plants and animals [16]. Thus, complementarity to 18S rRNA in IRES elements might recruit the 40S ribosomal subunit to initiate CIT [19]. The ribosome filter hypothesis has been proposed stating that ribosomal subunits themselves are regulatory elements for translation regulation of mRNAs [7]. From an evolutionary point of view, CIT in higher eukaryotes through 18S rRNA complementarity could be considered as a variant of the prokaryotic translation system. The leader of the cellular *Arabidopsis RPS18C* gene contains a 15-bp sequence with 100% complementarity to the 3′ end of the 18S rRNA at a position not previously identified in other transcripts. We showed IRES activity at the 84-bp leader containing this complementary element in an in vitro wheat germ extract translation system with dicistronic constructs. In addition, we provide experimental evidence that the 15-bp complementary element plays an important role in initiating the CIT. The finding of an element complementary to a novel position in the 18S rRNA with IRES activity indicates that CIT might require complementarity to 18S rRNA to recruit the 40S ribosomal subunit, but that its position is not critical, which is in accordance with the ribosome filter hypothesis [7].

The IRES database (www.iresite.org; [20]) lists IRES in several mammalian and one plant cellular mRNAs with a function in diverse cellular processes, such as stress response, response to hypoxia and ischemia, development, angiogenesis, cell cycle, oncogenesis, apoptosis, transcription and translation initiation control, and amino acid transport. Amongst these mRNAs are transcription factors, RNA processing factors, translation factors, kinases, channels, transporters, receptors, and growth factors. So far, no IRES activity has been reported in cellular ribosomal protein genes, although the coding sequence of the mouse S15 ribosomal protein gene contains a sequence complementary to the 18S rRNA [4]. Recently, the first plant cellular mRNA derived from the *HSP101* gene has been shown to have IRES activity at its leader [10]. Our data on IRES activity at the leader of the primary transcript derived from *RPS18C* adds evidence that also in plants 40S ribosome complementarity is used as a mechanism for translation regulation in addition to the cap-dependent translation process.

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